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PCR Amplification Of Trypanosoma Cruzi - Specific DNA from Raccoon Blood Samples

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PCR AMPLIFICATION OF *TRYPANOSOMA CRUZI* - SPECIFIC DNA FROM RACCOON BLOOD SAMPLES

A Capstone Experience/Thesis Project Presented in Partial Fulfillment of the Requirements for the Degree Bachelor of Sciences with Honors College Graduate Distinction at Western Kentucky University

By

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ABSTRACT

The parasitic protist *Trypanosoma cruzi* is the causative agent of Chagas Disease. Chagas Disease causes greater than 15,000 deaths each year, and nearly 28 million people are believed to be at risk of infection in Central and South America. This parasite has been described in many mammalian host species and has also been described in the United States. The purpose of this study was to attempt to use PCR to amplify *T. cruzi*-specific DNA directly from blood samples obtained from raccoons (*Procyon lotor*) trapped in Warren and Barren Counties of Kentucky in 2007 and 2008. DNA was successfully isolated from 487raccoon blood samples using a Qiagen QIAmp DNA Blood Mini Kit. Each DNA sample was then subjected to PCR amplification using the *T. cruzi* specific primer pair known as TCZ1 and TCZ2. It was determined that *T. cruzi* DNA was present in 47% of raccoon blood samples with a 57% prevalence in Warren county and a 32% prevalence in Barren county. Groce (2008) estimated a similar overall prevalence of 38% in these same raccoons based upon the results of hemocultures established at the time of sample collection.

Keywords: *Trypanosoma cruzi*, Chagas Disease, PCR, TCZ1, TCZ2, Gel Electrophoresis.

Dedicated to Mom, Dad, Sharon, Mike, Elizabeth, Patrick, Caleb, Marilyn, Chris, and to everyone who I could not list out by name here, but from whom blessings have flowed ceaselessly, and for which I have been entirely undeserving of.

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I would also like to thank former graduate students Chad Groce, Sanjay Varikuti, and Lipeng Bi. I would like to thank Chad Groce for his raccoon blood samples and hemoculture data that made up the bulk of the data for this study, and I would like to thank Sanjay Varikuti and Lipeng Bi for helping to educate me in the laboratory technique that I used for most of this study, and for simply being friendly and helpful faces in the laboratory when I first joined two and a half years ago. Without them, this study would have been impossible. I would also like to acknowledge that this research was funded in-part by NIH grant 2 P20 RR-16481.

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VITA

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FIELDS OF STUDY

Major Fields: Biology and Chemistry

Focus: Pre-med

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CHAPTER ONE

INTRODUCTION

Discovery of Chagas Disease:

While studying malaria in Brazil in 1909, Carlos Chagas was the first to discover the protozoan parasite, *Trypanosoma cruzi* (Chagas, 1909). Chagas noticed symptoms in the local Brazilian population that he could not attribute to malaria or to any other known disease. In his life, Chagas was able to determine not only the clinical symptoms of acute infection, but also the epidemiology and life cycle of the parasite within the vector and human host (Clayton, 2010 [A]). In honor of Carlos Chagas' extensive work, the disease is now known as Chagas Disease.

Chagas by the Numbers:

Chagas disease has taken the lives of countless people due to heart failure and other cardiological and digestive system maladies. The latest studies estimate that the disease causes approximately 12,500 to >15,000 deaths each year (Dias et al., 2008; Clayton, 2010 [A]). In addition, approximately 10-12 million people are living today who are infected with *T. cruzi* (Clayton, 2010 [A]), many of whom have no idea they carry the parasite. Studies have also shown that an estimated 28 million people are at risk of contracting the disease from the insect vectors, blood-sucking bugs in the family Reduviidae (Kirchhoff, 2003). In rural areas, these

triatome insects (commonly known as “kissing bugs”) reside in the cracks and crevices of palm trees, wood and rock piles, mud houses and livestock stables.

The acute stage of infection with *T. cruzi* presents with very general symptoms: headache, fever, swollen lymph nodes (lymphadenopathy), enlargement of the liver and spleen (hepatosplenomegaly), and inflammation of cardiac tissue (myocarditis; CDC DPDx, 2011). One telling clinical sign that can occur is known as a chagoma. A chagoma is a localized swelling at the site where the parasites enter the body, and the swelling can last for several weeks before finally subsiding (Rassi et al. 2010). When this chagoma is localized around the soft mucosa of the orbital region, it is referred to as Romaña’s sign. Romaña’s sign is the painless unilateral swelling of the periorbital area that occurs roughly 1 to 2 weeks after exposure (CDC DPDx, 2011). However, the acute phase of Chagas disease is not always clinically apparent, and in some cases, acute symptoms don’t even surface at all. The disease can progress to an asymptomatic (or latent) phase for 30+ years before it presents again in the chronic phase with cardiac abnormalities, megaesophagus or megacolon, and ends most notably in congestive heart failure and death (Coura and Vinas, 2010). Approximately 1/3 of patients who become infected with *T. cruzi* in endemic areas eventually develop the chronic manifestations of Chagas disease.

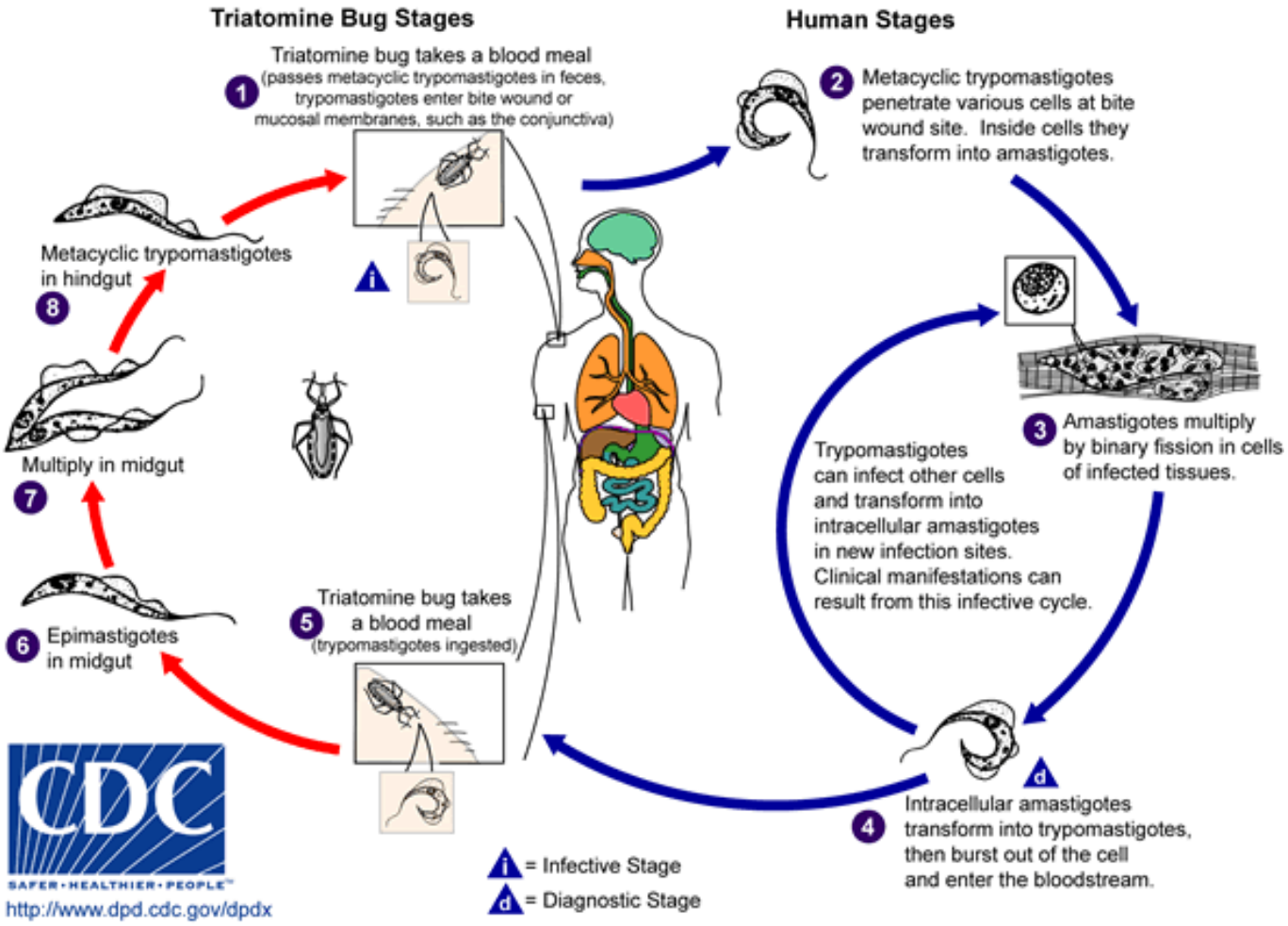
Life cycle of *T. cruzi*:

Triatome insects in the family Reduviidae are attracted to the CO_2 exhaled by vertebrates, and the bugs take blood meals by biting near the mouth and nose of the host, usually while the host sleeps (Harder, 2004). As it takes a blood meal, the

triatome bug often times defecates. Metacyclic trypomastigote stages of *T. cruzi* are present in the bug's feces, and it is this stage that is infective for the vertebrate host (CDC DPDx, 2011). The parasites enter broken skin (often at the site of the bite) or cross the soft mucosal tissues of the eyes, nose and lips (De Souza, 2002). Once inside the host, the trypomastigotes can enter into most types of tissue (Coura and Viñas, 2010; Clayton, 2010). After entering a host cell, parasites transform into the amastigote life stage and begin multiplying rapidly by binary fission (CDC 2011, CDC DPDx 2011). The amastigote stages reside within the cytoplasm of the host cell until transforming back into trypomastigote stages, or until the host cell dies and bursts expelling the multitude of amastigotes contained inside. This cycle continues in many different tissues throughout the body, and can do so for many days before the host experiences clinically apparent symptoms (Coura and Viñas, 2010).

The life cycle of the parasite continues when the host is again bitten by a "kissing bug". The bug ingests infected blood of the host animal or human, and within the midgut of the triatome insect, trypomastigote stages transform into epimastigote stages and reproduce asexually to form more metacyclic trypomastigotes. These stages then migrate to the hindgut of the insect and wait for defecation to occur. Once the triatome insect takes another blood meal, it defecates and the cycle begins anew (CDC DPDx, 2011). In addition, oral infection can occur by consumption of food or beverages contaminated with feces from infected triatome bugs. Recently, a total of 103 cases of Chagas disease were diagnosed in

Fig. 1- Summary of life cycle of *Trypanosoma cruzi* (CDC DPDx, 2011)



a school in Venezuela when children accidentally ingested contaminated guava juice (Clayton, 2010 [A]).

In endemic areas of Central and South America, the risk of infection from blood donations, blood transfusions, and organ donations is also high. In 1960, patients receiving infected blood transfusions and organ donations in Brazil's two largest cities acquired an estimated 16,000 cases of Chagas disease. The World Health Organization also estimated that roughly 7 million cases of Chagas were transmitted from infected blood and organs in the entire Latin American region in that same year (Coura and Viñas, 2010). Although screening and public awareness have improved significantly in many Latin American countries, the risk of transfusion and organ donation acquired infection remains unacceptably high in the region (Clayton, 2010 [B]; Coura and Viñas, 2010). People from Latin America who carry the disease frequently immigrate to foreign countries potentially unaware of the infection they carry. This has vast international implications for many governments and health care systems that have never before experienced or known about this parasite. Couras and Viñas (2010) recently reported that there are >300,000 individuals infected with *T. cruzi* in the United States, >5,500 in Canada, >80,000 in Europe and in the western Pacific region, >3,000 in Japan and >1,500 in Australia. With the world rapidly moving towards globalization, this raises the need for more education and screening for this parasitic infection worldwide.

Sylvatic cycle of *T. cruzi* in North America

Within the United States, the sylvatic cycle of infection with *T. cruzi* has been described and documented in the states of Alabama, California, Florida, Georgia, Louisiana, Maryland, Oklahoma, North Carolina, South Carolina, Tennessee, Texas, Virginia and Kentucky (McKeever et al., 1958; Olsen et al., 1964; John and Hoppe, 1986; Karsten et al., 1992; Yabsley & Noblet, 2002; Dorn et al, 2007; Hancock et al, 2005; Groce, 2008). The most recent study, conducted by Groce in 2008, reported a high prevalence of infection with *T. cruzi* in raccoons and opossums trapped in Warren and Barren Counties of south central Kentucky. Within the North American continent, *T. cruzi* has been identified in many mammalian hosts including raccoons *(Procyon lotor*), opossums (*Didelphis virginiana*), gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*), macaques (*Macaca silenus*), lemurs (*Lemur catta*), woodrats (*Neatoma magister*), armadillos (*Dasypus novemcincthus*), bats (*Eptiscus fuscus*), moles (*Neurotrichus gibbsii*), and dogs (*Canis familiaris*) (Yabsley et al., 2001; James et al., 2002; Hall et al., 2007). Despite infecting a wide variety of mammalian hosts in North America, the incidence of human infection remains low, with only seven confirmed autochthonous cases occurring within the United States (Bern et al., 2007). The rate of infection within the United States is so low presumably due to better living conditions and different feeding habits of the Triatome vector.

Detection of *T. cruzi*:

There have been several different methods employed to detect *T. cruzi* in samples of blood, including direct microscopy, xenodiagnosis, hemoculture, and polymerase chain reaction (PCR). The PCR method utilizes gene specific oligonucleotide primers that target a distinct gene sequence within a sample, and amplifies that gene sequence potentially hundreds of thousands of time. These thousands of copies of the DNA sequence (known as amplicons or PCR products) can then be visualized following agarose gel electrophoresis to determine the presence/absence of the target sequence within the sample. Studies have proven that PCR is far more sensitive than microscopic methods (Kirchhoff et al., 1996), especially when using the TCZ1 and TCZ2 primer pair (Moser et al., 1989). The TCZ1 and TCZ2 primers are specific for a highly conserved 195-base pair (bp) repetitive nuclear sequence of *T. cruzi* (Moser et al., 1989). The goal of the present study was to attempt to use PCR to amplify *T. cruzi*-specific DNA directly from blood samples obtained from raccoons (*Procyon lotor*) trapped in Warren and Barren Counties of Kentucky in 2007 and 2008. We hypothesize that PCR analysis will allow for a more accurate determination of the prevalence of *T. cruzi* infection in raccoons than the hemoculture method previously employed by Groce in 2008.

CHAPTER 2

MATERIALS AND METHODS

DNA Extraction:

200 µL of whole, non-coagulated blood samples stored at 4°C from 47 raccoon specimens were used in this study. DNA was extracted using the Qiagen QIAamp® DNA blood mini kit, according to the manufacturer's recommended protocol. Afterwards, DNA concentration was determined using a Thermo Nanodrop ND-100 spectrophotometer. Purified DNA samples were stored at -20°C until use.

PCR Amplification:

PCR amplification was performed on each DNA sample, using the TCZ1 and TCZ2 primer set. The TCZ1 and TCZ2 primer pair was designed to amplify 188 bp of a 195-bp repetitive nuclear sequence of *T. cruzi* (Moser et al., 1989). The TCZ1 and TCZ2 primers consisted of the following nucleotide sequences: TCZ1 (5'-CGA GCT CTT GCC CAC ACG GGT GCT 3') TCZ2 (5'-CCT CCA AGC AGC GGA TAG TTC AGG 3'). For each PCR analysis, a 50µl reaction was set up containing 20µl 2.5X Master Mix (5 Prime, Gaithersburg, MD), 1 µl (4µM) of each primer, a standardized amount of template DNA, and sterile nanopure water to bring the total volume to 50 µl. A negative control containing sterile nanopure water in place of template DNA was included with each analysis. Two positive controls using template DNA

prepared from *T. cruzi* type I and type IIa reference strains were also included. Reaction mixtures were loaded into an automated DNA thermal cycler to undergo amplification according to the protocol outlined in Table 1. The TCZ1 and TCZ2 primers are highly specific for *T. cruzi*-DNA, and should not amplify other DNA that is found within the blood samples (i.e. raccoon leukocyte DNA).

Table 1. Thermocycler Protocol used for TCZ1 and TCZ2 primers.

Steps	Temperature (°C)	Time	# of cycles
Initial Denature	94	2 min	1
Denature	94	1 min	30
Annealing	64.5	30 sec	
Extension	72	15 sec	
Final extension	72	5 min	1
Hold	4	Indefinite	1

Agarose Gel Electrophoresis:

Once all of the samples had been subjected to the PCR procedure, a 2 µL sample of the resulting PCR products were stained with a phosphorescent dye and loaded onto 3% agarose gels and subjected to electrophoresis at 80V for 2 hours to separate the amplified DNA fragments. The resulting gels were imaged using a Fluorochem HD2 ultraviolet imager. A 50bp DNA ladder was employed to allow an estimate of the length of our target DNA sequence. Additionally, the PCR procedure was repeated on all samples, to confirm results.

After gels were analyzed and the positive and negative samples were determined, results were compared to the 2008 study performed by Groce, to determine how PCR analysis performed in comparison to hemoculture.

CHAPTER 3

RESULTS

After total DNA was isolated from each blood sample using the Qiagen QIAamp® DNA blood mini kit, DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer. Concentrations ranged from 8.6 ng/µL to 210.9 ng/µL, with an average concentration of between 30 and 50 ng/µL. These values are shown in Tables 1 and 2 below.

Following PCR amplification of all DNA samples, products were analyzed by agarose gel electrophoresis. Twenty-two out of 47 DNA samples were judged to be PCR positive for *T. cruzi* DNA based on the presence of a 188 bp band visible in the gel with UV illumination. The results of PCR analysis on Warren County raccoon DNA samples are shown in Figures 2a and 2b and the results of PCR analysis on Barren County raccoon DNA samples are shown in Figure 3. Results are also summarized in Tables 2 and 3.

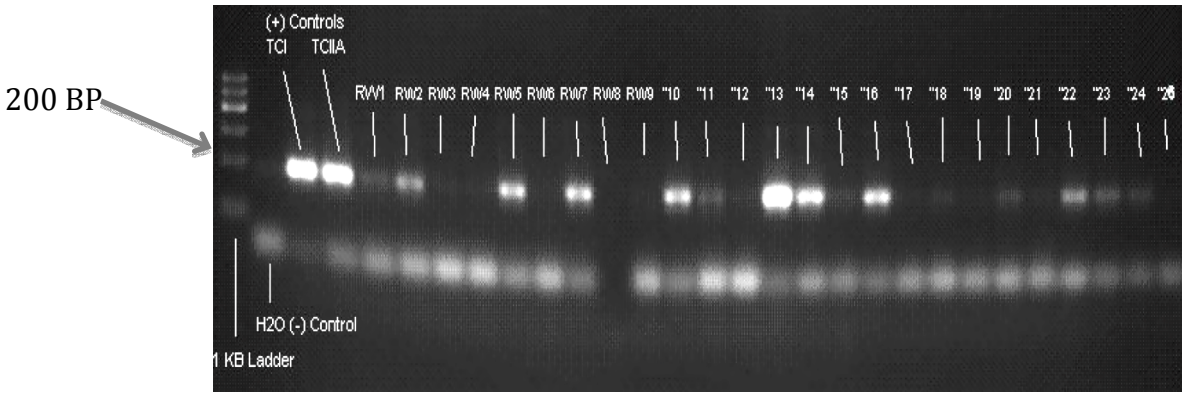


Figure 2A. PCR products resulting from amplification of raccoon DNA (RW series; part I) with primers TCZ1 and TCZ2 followed by agarose gel electrophoresis (3% gel).

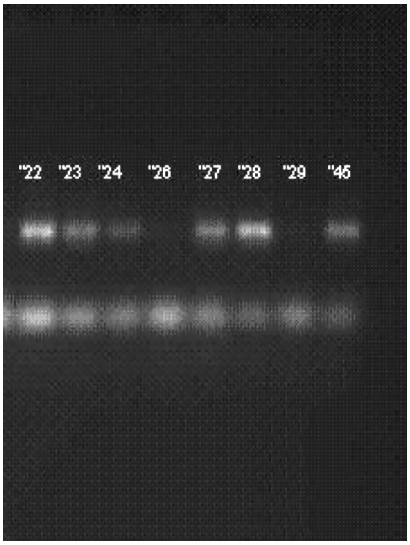


Figure 2B. PCR products resulting from amplification of raccoon DNA (RW series; part II) with primers TCZ1 and TCZ2 followed by agarose gel electrophoresis (3% gel).

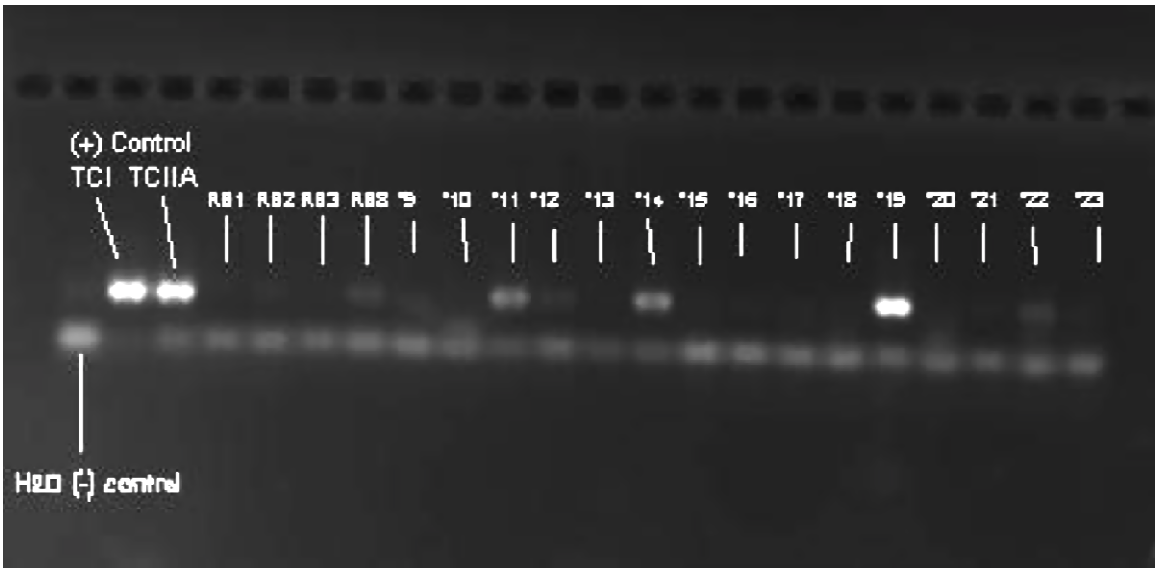


Figure 3. PCR products resulting from amplification of raccoon DNA (RB series) with primers TCZ1 and TCZ2 followed by agarose gel electrophoresis (3% gel)

Gel electrophoresis was repeated on the samples that were faintly positive or samples that were judged to be positive by hemoculture or by prior PCR analysis. The results of this second electrophoretic analysis can be seen in Figure 4. Hemoculture results versus PCR positive samples are shown in Tables 4 and 5. In all, there were 7 additional positive samples in which the sample was PCR positive and hemoculture negative in our data, and 3 discrepancies where the hemoculture showed positive results and the PCR negative.

Tables 2 and 3: Sample number, concentration, and presence of *T. cruzi* DNA for Warren and Barren counties, respectively.

Sample Number (Warren Series)	DNA Concentration (ng/µL)	Presence of *T. cruzi* DNA?
RW1	67.4	+
RW2	33.3	+
RW3	17.5	-
RW4	34.6	-
RW5	53.9	+
RW6	36.5	-
RW7	31.2	+
RW8	33.6	-
RW9	12.9	-
RW10	35.5	+
RW11	78.7	+
RW12	8.6	-
RW13	155.3	+
RW14	110.7	+
RW15	29.3	+
RW16	32.7	+
RW17	30.3	-
RW18	28.2	-
RW19	32.4	-
RW20	33.6	+
RW21	42.7	-
RW22	36.7	+
RW23	28.7	+
RW24	28.7	+
RW26	77.7	-
RW27	45.1	+
RW28	38.2	+
RW29	48.4	-

Sample Number (Barren Series)	DNA Concentration (ng/µL)	Presence of *T. cruzi* DNA?
RB1	21.0	-
RB2	14.4	-
RB3	10.0	-
RB8	12.0	+
RB9	44.3	-
RB10	45.4	-
RB11	32.6	+
RB12	17.5	+
RB13	210.9	-
RB14	32.7	+
RB15	14.7	-
RB16	21.3	-
RB17	35.3	-
RB18	46.1	-
RB19	40.8	+
RB20	38.4	-
RB21	42.4	-
RB22	31.4	+
RB23	66.5	-

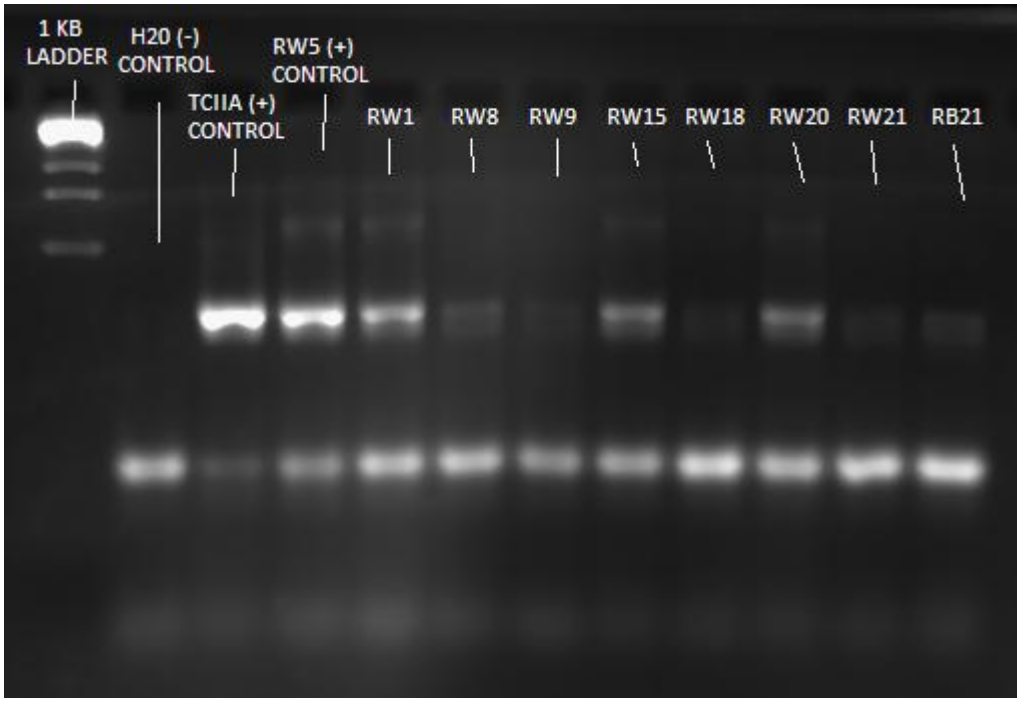


Figure 4. PCR products resulting from amplification of selected raccoon DNA samples from the RW and RB series with primers TCZ1 and TCZ2 followed by agarose gel electrophoresis (3% gel).

Tables 4 and 5: The 2008 Hemoculture versus present PCR data for the Warren and Barren county samples, respectively. Note: The light gray shading denotes a negative (-) hemoculture result and a positive (+) PCR result. The dark gray shading denotes a positive (+) hemoculture result and a negative (-) PCR result.

Sample	Hemoculture	PCR Results
RW1	-	+
RW2	+	+
RW3	-	-
RW4	-	-
RW5	+	+
RW6	-	-
RW7	+	+
RW8	-	-
RW9	+	-
RW10	+	+
RW11	+	+
RW12	-	-
RW13	+	+
RW14	+	+
RW15	+	+
RW16	-	+
RW17	-	-
RW18	+	-
RW19	-	-
RW20	+	+
RW21	+	-
RW22	+	+
RW23	-	+
RW24	+	+
RW26	-	-
RW27	+	+
RW28	-	+
RW29	-	-

Sample	Hemoculture	PCR results
RB1	-	-
RB2	-	-
RB3	-	-
RB8	-	+
RB9	-	-
RB10	-	-
RB11	+	+
RB12	+	+
RB13	-	-
RB14	+	+
RB15	-	-
RB16	-	-
RB17	-	-
RB18	-	-
RB19	-	+
RB20	-	-
RB21	-	-
RB22	-	+
RB23	-	-

CHAPTER 4

DISCUSSION

The results of the present study confirm a high prevalence of *T. cruzi* infection in raccoon populations in Warren and Barren counties of Kentucky. Direct PCR amplification of raccoon blood samples using the highly sensitive *T. cruzi*-specific primers TCZ1 and TCZ2 revealed that 57% (16/28) of Warren county raccoons were positive for *T. cruzi* DNA and 32% (6/19) of raccoon blood samples from Barren County were positive. A total of 22 out of 47 raccoons in this study were judged to contain *T. cruzi* DNA, giving an overall prevalence of 47% for the region.

A variety of studies have been performed to determine the prevalence of *T. cruzi* in raccoon populations in various regions of the United States over the past several decades. The prevalence of infection reported in these studies ranged from a low of 1.5% reported by Mckeever and coworkers in Georgia and Florida in 1958 (Mckeever et al., 1958) to a high of 63% reported in a study performed by John and Hoppe in Oklahoma in 1986. However, the John and Hoppe study did have a very small sample size with only 8 raccoon samples collected (John and Hoppe, 1986). Since many of these studies have been performed, the scientific community has seen the advent of newer and more sensitive diagnostic methods such as hemoculture,

indirect immunofluorescence assay (IFAT), enzyme-linked immunosorbent assay (ELISA) and PCR which have made testing for the parasite much more efficient and sensitive than the archaic methods of direct visual microscopy and xenodiagnosis.

Every method of parasite detection that has been developed has its advantages and disadvantages, yet PCR is widely viewed as one of the most sensitive and effective procedures currently available. Visual microscopy of blood smears or tissue mounts is the most direct method of identification, however, microscopy has its disadvantages because the blood or tissues biopsied may not contain the parasites, the blood and tissues must be fresh in order to preserve the parasite for identification, and this method is time consuming and does not allow for as many samples to be processed daily. In contrast, PCR can amplify incredibly small amounts of DNA within a sample, it can be performed on an older sample (as demonstrated by this study), and large numbers of samples can be analyzed simultaneously. PCR also has been shown to be significantly more effective at *T. cruzi* identification than direct microscopy (Pizarro et al. 2007). The technique known as xenodiagnosis is a longstanding indirect method of detecting *T. cruzi*, however xenodiagnosis can also fail since the triatome insects do not always ingest the parasite, and the parasite may not always proliferate within the gut as expected. This technique also requires the labor-intensive maintenance of large quantities of triatome bugs in the laboratory. Hemoculture requires blood samples to be introduced into culture medium under sterile conditions, with hopes that the parasite will eventually grow and be identified. However, as with other methods,

the samples must be fresh and free of bacterial contamination, and the parasites may not grow as expected. Finally, IFAT is also an effective method of diagnosis, but it too can be inaccurate because it relies on the immunological response of the host (raccoon, human). The IFAT can give a false negative result if for some reason the host is immunologically compromised or if the blood sample is taken too early in the infection process. The PCR technique is much more effective because it tests directly for the presence of the parasitic DNA within the sample. It also doesn't depend upon the immune response of the host (as with IFAT), or the time consuming process of in vitro cultivation under sterile conditions (hemoculture). The PCR technique does depend upon the effectiveness of the chosen primers and the quality of the DNA samples, but if these conditions are met, PCR can be extremely specific and sensitive.

The TCZ1 and TCZ2 primers used in this study were carefully chosen for those exact reasons. The TCZ primer pair recognizes a highly repetitive, non-coding, nuclear sequence within the *T. cruzi* genome (Moser et al., 1989; Kirchhoff et al., 1996). Due to the highly repetitive nature of this sequence within the genome, amplification occurs at a much higher rate than if the primers were recognizing just one region of DNA. The TCZ1 and TCZ2 primers are also highly specific and can only recognize the target DNA sequence and not any other DNA present in the sample. The selection of the TCZ1 and TCZ2 primers for this study was based upon a careful review of the literature. Many other studies have shown these primers to be effective in various situations including identification in insect vectors and reservoir

hosts (McKeever et al., 1958; Olsen et al., 1964; John and Hoppe, 1986; Karsten et al., 1992; Yabsley & Noblet, 2002; Dorn et al, 2007; Hancock et al, 2005; Groce, 2008, Yabsley et al, 2001; Hall et al, 2007) and in patients with Chagas disease (Avila et al., 1993; Dorn et al., 2007; Bern et al., 2007;G. Russomando, et al. 1992; G. Russomando et al. 1998; Leiby, et al. 2000; Maldonado et al., 2004; Carriazo et al., 1998; Marcon et al., 2011). There have also been studies performed on very old or archived specimens where it was shown that PCR was still effective in detecting *T. cruzi* long after any other method would have been unusable (James et al., 2002; Williams et al., 2009). For example, TCZ1 and TCZ2 primers were used in a 7 years post-mortem patient to determine the exact cause of death by Chagas disease (Ochs et al., 1996). Studies designed to compare the TCZ 1 and 2 primers with other primer sets have shown that the TCZ series is by far the most sensitive. In a 2005 study, Virreria and co-workers tested the TCZ1/TCZ2 primers against nuclear DNA primers BP1/BP2, O1/O2, Pon1/Pon2, and Tca1/Tca2 and kinetoplast DNA primers S35'/S36' and 121/122 and showed that TCZ1/TCZ2 primers were the most sensitive (Virreria et al., 2005). A study in 2006 by Trejo tested the TCZ series against the kinetoplast DNA primers S35'/S36' and 121/122 and reported the same findings (Trejo, 2006).

When the PCR results obtained in the present study were compared to the hemoculture results obtained by Groce in 2008, several discrepancies were observed. As seen in Tables 4 and 5, there were 7 samples that were originally hemoculture negative, but were determined to be PCR positive in this study (RW1,

RW16, RW23, RW28, RB8, RB19, and RB22). In addition, there were 3 samples that were hemoculture positive and PCR negative in the present study (RW9, RW18, RW21). In the cases where this study showed the RW1, RW16, RW23, RW28, RB8, RB19, and RB22 blood samples to be *T. cruzi* positive, we can assume that PCR was the more sensitive of the two methods in detecting the presence of the parasite. However, in three cases (RW9, RW18, and RW21) it would appear that hemoculture was more sensitive than PCR. One factor to consider when interpreting these results is the age of the blood samples at the time of DNA isolation. When this study first began in 2009, the raccoon blood samples had been stored at 4°C for over a year. Many investigators have confirmed that PCR can still be effective even years after samples would be considered to be non viable (James et al., 2002; Williams et al., 2009; Ochs et al., 1996). However, it seems reasonable to predict that the integrity of the DNA present in the raccoon blood samples would decrease with extended storage at 4°C. The only way this could have been avoided would have been to isolate DNA from the blood samples very soon after the raccoons had been trapped. What can be understood is that in the case of future studies for *T. cruzi* prevalence, multiple detection methods should be employed to get the most accurate results.

In conclusion, PCR has confirmed a high prevalence of *T. cruzi* infection in the raccoon populations of Warren and Barren counties in south central Kentucky. Our results demonstrate that PCR with the TCZ1 and TCZ2 primer pair can be a very effective method for detecting *T. cruzi* DNA in raccoon blood samples even after long-term storage at 4°C. However, to achieve the most accurate estimate of

parasite prevalence in wild mammal populations it is recommended that investigators use a combination of both hemoculture and PCR analysis.

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